

Candida queiroziae sp. nov., a cellobiose-fermenting yeast species isolated from rotting wood in Atlantic Rain Forest

Renata O. Santos · Raquel M. Cadete · Fernanda Badotti ·
Adriane Mouro · Daniela O. Wallheim · Fátima C. O. Gomes ·
Boris U. Stambuk · Marc-André Lachance · Carlos A. Rosa

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Abstract Eight strains of a novel yeast species were isolated from rotting wood and wood-boring insects in Atlantic Rain Forest ecosystems in Brazil. Sequences of the D1/D2 domains of the large subunit of the rRNA gene showed that the yeast belongs to the *Scheffersomyces* clade and that it is related to *Candida lignicola* and *Candida coipomoensis*. The new species was isolated from rotting wood of three different localities and a wood-boring insect suggesting that these substrates are its ecological niche. This new yeast species is able to assimilate cellobiose and other compounds related to rotting wood. Strong fermentation of cellobiose in Durham tubes was observed for the

strains of this new yeast. The new species produced an intracellular β -glucosidase responsible for cellobiose hydrolysis. The novel species, *Candida queiroziae* sp. nov., is proposed to accommodate these isolates. The type strain of *C. queiroziae* is UFMG-CLM 5.1^T (=CBS 11853^T = NRRL Y-48722^T).

Keywords New yeast species · *Candida queiroziae* · Cellobiose-fermenting yeast · Rotting wood · Atlantic Rain Forest

Introduction

Discarded cellulosic biomass derived from forestry, agriculture, and municipal sources are potential feed stocks for the production of bio-fuels, in particularly for the synthesis of fuel ethanol (Hahn-Hägerdal et al. 2006). Although cellulose is the most abundant biopolymer in the world, its chemical structure makes it difficult to hydrolyze (Zhu et al. 2010) and current strategies to produce fuel ethanol from cellulose should favour the simultaneous saccharification and fermentation of the substrate (Lynd et al. 2002, 2005). The process requires pre-treatment of the cellulosic feedstock by stem-explosion or acid treatment, followed by addition of exogenously produced cellulolytic enzymes to hydrolyze cellulose chains and release the monomers required for fermentation (Carere et al. 2008).

During the simultaneous saccharification and fermentation process, cellulose is hydrolyzed by the

R. O. Santos · R. M. Cadete · F. Badotti ·
C. A. Rosa (✉)
Departamento de Microbiologia, ICB, C.P. 486,
Universidade Federal de Minas Gerais,
Belo Horizonte, MG 31270-901, Brazil
e-mail: carlrosa@icb.ufmg.br

F. Badotti · F. C. O. Gomes
Departamento de Química, Centro Federal de Educação
Tecnológica de Minas Gerais, Belo Horizonte,
MG 30480-000, Brazil

A. Mouro · D. O. Wallheim · B. U. Stambuk
Departamento de Bioquímica, Centro de Ciências
Biológicas, Universidade Federal de Santa Catarina,
Florianópolis, SC 88040-900, Brazil

M.-A. Lachance
Department of Biology, University of Western Ontario,
London, ON N6A 5B7, Canada

cellulase enzyme complex (cellobiohydrolases, endoglucanases and β -glucosidases) working in synergy. Endoglucanases (EC 3.2.1.4) randomly cleave the β -1,4 glycosidic linkages of cellulose, cellobiohydrolases (EC 3.2.1.91) attack cellulose chain ends to produce the constitutive unit of cellulose, cellobiose, a disaccharide of glucose linked by a β -1,4 glycosidic bond, and finally β -glucosidases (EC 3.2.1.21) hydrolyze cellobiose into two glucose moieties (Kumar et al. 2008). The use of microorganisms able to ferment cellobiose constitutes a desirable alternative for bio-ethanol production because cellobiose is a competitive inhibitor of cellulases (Bezerra and Dias 2005). These strains also ferment the monomers produced during cellulose hydrolysis, and cellulases are thus not inhibited (Lynd et al. 2005).

In a study on yeast communities associated with rotting wood and wood-boring insects in Atlantic Rain Forest ecosystems in Brazil, we have isolated eight strains of a new asexual cellobiose-fermenting yeast species. Analysis of the sequence of the D1/D2 domains of the large subunit rRNA gene showed that these strains represented a single species closely related to *Candida lignicola* and *C. coipomoensis*. In this work, we describe this new species as *Candida queiroziae* sp. nov.

Materials and methods

Yeast isolation and identification

Five strains were isolated from rotting wood and one from a wood-boring insect collected in the Private Natural Heritage Reserve of the Sanctuary of the Caraça. This is an ecological reserve with 11,233 ha of Atlantic Rain Forest located in the Serra do Espinhaço, state of Minas Gerais, Southeastern Brazil (Cadete et al. 2009). Two other yeast strains were isolated from rotting wood samples collected at the Private Natural Heritage Reserves of Woodstock and Bello & Kerida, respectively, two areas of Atlantic Rain Forest ecosystems located in the city of Nova Friburgo, Rio de Janeiro state, Brazil. The local climate in these ecological reserves is altitudinal tropical, with cold and dry winter and fresh and rainy summers, with annual mean temperatures around 16°C.

Fifteen rotting wood and 16 wood-boring insect samples were collected from the Caraça Ecological

Park in April 2008. Ten samples each were collected from the Ecological Reserves of Woodstock and Bello & Kerida in January 2009. Rotting wood samples were stored in sterile plastic bags and transported under refrigeration to the laboratory over a period of no more than 24 h. Insects were stored in sterile flasks and transported under refrigeration to the laboratory as described by Suh et al. (2003). One gram of each wood sample was placed separately in flasks with 20 ml sterile D-xylose (yeast nitrogen base 0.67%, D-xylose 0.5%, chloramphenicol 0.02%) and carboxymethylcellulose (Yeast Nitrogen Base 0.67%, carboxymethylcellulose 1%, cellobiose 0.05% and chloramphenicol 0.02%) minimal media. The flasks were incubated at 25°C on an incubator shaker (New Brunswick, USA) at 150 rpm for 3–10 days. When growth was detected, 0.5 ml of the cultures was then transferred separately to tubes containing 5 ml sterile carboxymethylcellulose or D-xylose media and the tubes were incubated as described above. For yeast isolation from insects 100 μ l of a solution of insect gut macerate was inoculated in the same media and conditions mentioned above. One loopful of culture from each tube was streaked on yeast extract-malt extract agar (YM, glucose 1%, yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, agar 2% and chloramphenicol 0.02%). The plates were incubated at 25°C until yeast colonies developed. The different yeast morphotypes were purified by repeated streaking on YM agar plates and preserved at –80°C or in liquid nitrogen for later identification. The yeasts were characterized by standard methods (Yarrow 1998) and identification followed the taxonomic keys of Kurtzman and Fell (1998).

The ability to ferment cellobiose was tested in Durham tubes containing fermentation basal medium with a final sugar concentration of 2% (w/v) (Yarrow 1998). The tubes were incubated at 25°C on an incubator shaker (New Brunswick) at 100 rpm for 25 days.

DNA sequencing and sequence analysis

The D1/D2 variable domains and internal transcribed spacer (ITS) of the large subunit rRNA gene were amplified by PCR directly from whole cells as described previously (Lachance et al. 1999). The amplified DNA was concentrated, cleaned (Wizard Plus SV Minipreps DNA Purification System, Promega) and sequenced in a Mega-BACETM 1000

automated sequencing system (Amersham Biosciences) in Institute of Biological Sciences of the Federal University of Minas Gerais, Brazil, or sequenced at the Robarts Research Institute, London, Ontario, in Canada. The sequences were edited with the program DNAMAN, version 6 (Lynnon BioSoft, Vaudreuil, QC, Canada). Existing sequences for other yeasts were retrieved from GenBank. The CLUSTAL W software (Thompson et al. 1994) included in DNAMAN was used to align the D1/D2 LSU rRNA gene sequences. The alignment covered 534 positions, of which 63 were polymorphic, 38 were parsimony-informative and 13 were gapped. For tree construction using the built-in Neighbour-Joining algorithm, gapped positions were deleted in pairs and Kimura's two-parameter distance was applied.

Growth conditions and fermentation assays

Cells were grown on YP medium (1% yeast extract and 2% peptone), adjusted to pH 5.0, supplemented with 2% glucose or cellobiose. Cells were grown with shaking at 28°C (160 rpm) in cotton-plugged Erlenmeyer flasks filled to 1/5 of the volume with medium. The inoculum for growth assays was prepared by transferring a single colony aseptically from a plate into 5 ml of the YP medium containing glucose or cellobiose, and allowing growth to proceed to the stationary phase for 2–3 days before inoculating cells (by a 100 or 1,000× dilution factor) to new media of a similar composition. Culture samples were harvested regularly, centrifuged (5000×g, 1 min) and their supernatants were used for the determination of sugars and ethanol. For batch fermentations, the yeasts were pre-grown on YP-2% sugar to the late exponential phase (~1 g dry yeast l⁻¹), centrifuged (3,500×g, 3 min), washed twice with cold water and inoculated at a high cell density (10 g dry yeast l⁻¹) into YP medium containing the amounts of glucose and/or cellobiose indicated. Batch fermentations were incubated as described above for growth assays, and samples were collected regularly, centrifuged and their supernatants were analyzed as described below.

Determination of β -glucosidase activity

Periplasmic or secreted cellobiose hydrolysis by β -glucosidase was determined in vivo with whole

cells previously incubated with 50 mM sodium fluoride to block glycolysis (Silveira et al. 1996), using 67 mM cellobiose in either 67 mM succinate–Tris, pH 5.0, or 67 mM HEPES–NaOH, pH 7.0. Intracellular β -glucosidase activity was determined in situ with permeabilized yeast cells (Stambuk 1999) using 100 mM cellobiose in either 100 mM succinate–Tris, pH 5.0, or 100 mM HEPES–NaOH, pH 7.0. Controls using previously boiled cells were used. Enzyme activity is expressed as U/g dry yeast cells, where one unit corresponds to 1 μ mol of glucose produced/min at 28°C.

Analytical methods

Glucose was measured by the glucose oxidase and peroxidase method using a commercial kit (BioDiagnostica-Laborclin, Brazil), and cellobiose was determined as described by Miller (1959). Ethanol was determined with alcohol oxidase (Sigma) and peroxidase (Toyobo do Brasil, Brazil) as described previously (Alves et al. 2007). Growth was followed by turbidity measurements at 570 nm after appropriate dilution, and yeast cell dry weight was determined as described elsewhere (Badotti et al. 2008). Ethanol yield coefficients ($Y_{e/s}$) were obtained at the end of ethanol production taking into account the amount of sugar utilized.

Results and discussion

The sequences of the D1/D2 domains and ITS region of the large subunit rRNA gene of the eight strains of the new species were identical. This new species belongs to the *Scheffersomyces* clade and it is closely related to *C. lignicola* and *C. coipomoensis* (Fig. 1). The genus *Scheffersomyces* was proposed by Kurtzman and Suzuki (2010) to include several D-xylose-fermenting yeast species related to *Pichia stipitis*. This clade contains the species *S. stipitis*, *S. segobiensis*, *S. spartinae* and the anamorphic yeasts *C. lignosa*, *C. insectosa*, *C. shehatae*, *C. coipomoensis*, *C. lignicola* and *C. ergastensis*. The new species differs by 11 substitutions and one gap from *C. lignicola*, and 13 substitutions from *C. coipomoensis* in the D1/D2 domains, and by eight indels from *C. lignicola* and six substitutions from *C. coipomoensis* in the ITS region of

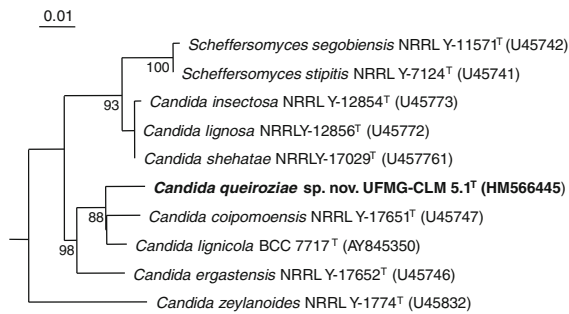


Fig. 1 Neighbour-joining tree of the D1/D2 domains of the large subunit of the rRNA gene showing the placement of *Candida queiroziae* sp. nov. among related species in the *Scheffersomyces* clade. Bootstrap values of 50% and above are shown. *Candida zeylanoides* NRRL Y-1774 is the outgroup species, and all species are analyzed from type strains. Bar 0.01 substitutions per nucleotide position

the large subunit rRNA gene. The name *C. queiroziae* sp. nov. is proposed for the new species.

The strains of the new species were isolated from different sources and localities. The new species is not able to ferment D-xylose, but strong fermentation of cellobiose in Durham tubes was observed after 24 h for strain UFMG-IMX 6.1 isolated from a boring wood insect and after 3–6 days for the yeast strains isolated from wooding root samples (Table 1). The strains of *C. queiroziae* are physiologically similar to *C. lignicola* and *C. coipomoensis*. *C. lignicola* is able to assimilate L-arabinose and L-rhamnose, which does not occur with the new species. *C. queiroziae* can be distinguished from *C. coipomoensis* based on the assimilation of D-Gluconate, which is positive for the new species and negative for *C. coipomoensis*. In addition, *C. queiroziae* is able to grow on 10% NaCl plus 5% glucose medium, whereas the other two species did not grow. Isolates of *C. queiroziae* were examined individually or mixed in pairs on cornmeal, V8, dilute V8, 5% malt extract, yeast carbon base supplemented with 0.01% ammonium sulphate, and Gorodkova agars incubated at 20 or 28°C until 30 days, but asci or signs of conjugation were not seen.

Candida coipomoensis was isolated from wood in advanced stages of degradation in the evergreen rainy Valdivian forest of Southern Chile (Ramirez and González 1984), and *C. lignicola* was isolated from insect frass collected from Khao-Yai National Park of Thailand (Jindamorakot et al. 2007). Similar to their closest relatives, *C. queiroziae* was isolated from

rotting wood of three different localities and a wood-boring insect suggesting that these substrates are the ecological niche of this new species.

Growth and fermentation of cellobiose by *Candida queiroziae*

Candida queiroziae is able to assimilate cellobiose and other compounds related to rotting wood, and tests in Durham tubes indicates that this yeast species can ferment cellobiose within variable periods of time (see Table 1). Figure 2 shows the kinetics of growth on cellobiose by the strain UFMG-CLM 5.1^T and UFMG-IMX 6.1. Both strains exhibited a typical growth curve where the sugar is efficiently fermented. After the sugar is exhausted from the medium, the ethanol produced starts to be consumed and used as a carbon source by the yeast. In agreement with the fermentation results in Durham tubes (Table 1), strain UFMG-IMX 6.1 grew and consumed cellobiose faster than strain UFMG-CLM 5.1, although at the end of the fermentation both strains produced practically the same amount of biomass (Fig. 2). An ethanol yield from cellobiose ($Y_{e/s} = 0.32 \pm 0.03$) obtained with these two strains of *C. queiroziae* is in the same range as those obtained during glucose fermentation ($Y_{e/s} = 0.32 \pm 0.01$) by these yeasts (data not shown). Although during growth strain UFMG-IMX 6.1 apparently utilized cellobiose more efficiently than strain UFMG-CLM 5.1, during batch fermentations at high cell densities (Fig. 3) no significant differences in the fermentation of glucose (Fig. 3a) or cellobiose (Fig. 3b) between these two strains was observed, with cellobiose fermentation by both strains occurring at a slightly slower rate than glucose fermentation.

The lack of glucose production during growth on cellobiose (Fig. 2b), or even during batch fermentation of this sugar (Fig. 3b), prompted us to characterize in more detail the subcellular localization (periplasmic or intracellular) of the β -glucosidase responsible for cellobiose hydrolysis by *C. queiroziae*. Our results indicate that both strains UFMG-IMX 6.1 and UFMG-CLM 5.1 lack (or have very low) periplasmic β -glucosidase activity (Fig. 4), with rates of cellobiose hydrolysis of less than ~ 5 U/g dry yeast cells at either pH 5.0 or 7.0. When the yeast cells were permeabilized, a significant β -glucosidase activity could be seen both at pH 5.0 (29–47 U/g dry yeast cells), and especially at pH 7.0 (167–230 U/g dry

Table 1 Source, origin, culture media for isolation and days for cellobiose fermentation of the strains of *Candida queiroziae*

Strains	Source	Origin (Atlantic Rain Forest ecosystem)	Culture media	Cellobiose fermentation (days to fill the Durham tube)
UFMG-CLM 5.1, UFMG-CLM 8.1,	Rotting wood	Sanctuary of the Caraça.	Carboxymethylcellulose	4
UFMG-CLM 6.1, UFMG-CLM 12.1	Rotting wood	Sanctuary of the Caraça.	Carboxymethylcellulose	3
UFMG-CLM 7.2	Rotting wood	Sanctuary of the Caraça.	Carboxymethylcellulose	6
UFMG-IMX 6.1	Wood-boring insect	Sanctuary of the Caraça.	D-xylose	1
UFMG-CLM 43.1	Rotting wood	Woodstock	Carboxymethylcellulose	3
UFMG-CLM 57.2	Rotting wood	Bello & Kerida	Carboxymethylcellulose	3

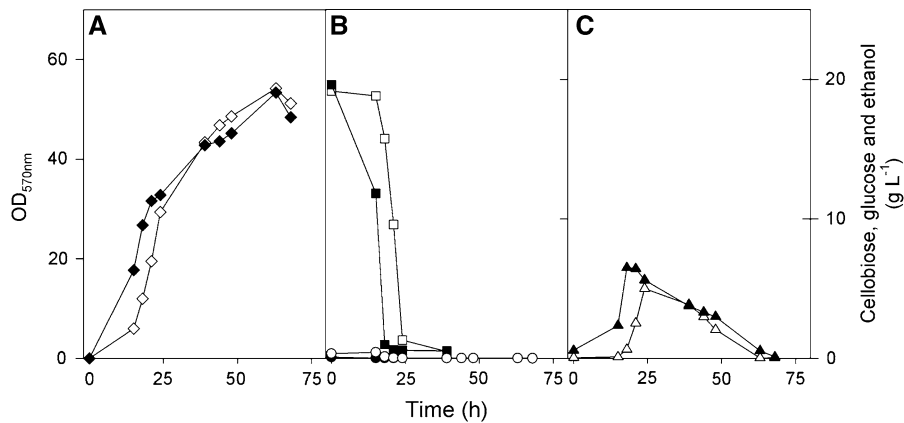


Fig. 2 Typical aerobic batch growth on cellobiose by *Candida queiroziae* UFMG-CLM-5.1^T (open symbols), or strain UFMG-IMX-6.1 (black symbols). **a** Cell density (diamonds), **b** cellobiose (squares) consumption, and **c** production of ethanol

(triangles), were determined during growth in rich YP medium containing 20 g l⁻¹ of cellobiose. In **b** are also shown glucose (circles) concentrations in the medium during cellobiose utilization

yeast cells) by both strains, which is consistent with an intracellular β -glucosidase as being responsible for cellobiose hydrolysis by *C. queiroziae*. While yeast species (e.g. *Saccharomyopsis fibuligera*, *Wickerhamomyces anomalus* and *C. wickerhamii*) ferment cellobiose by expressing β -glucosidases at the cell surface, very few yeast are reported as capable of fermenting this sugar through an intracellular enzyme, a process which requires the uptake of the disaccharide into the cell. Unfortunately, when compared to our current knowledge of other disaccharide uptake system in several yeast species, cellobiose transport systems have been poorly characterized (Freer and Greene 1990; Spencer-Martins 1994). In view of its efficient cellobiose fermentation *C. queiroziae* may provide a new source of genes, enzymes and/or sugar transporters to engineer industrial strains for efficient

ethanol production from renewable biomass (Galazca et al. 2010; Li et al. 2010).

Latin diagnosis of *Candida queiroziae* Santos, Cadete, Badotti, Mouro, Wallheim, Gomes, Stambuk, Lachance & Rosa sp. nov.

In medio liquido post dies tres (25°C) cellulae singulae aut binae; cellulae ovoidae aut ellipsoideae (2–3 × 2–4 μm). Post unum mensem sedimentum formatur. Cultura in agaro malti post dies 2 (17°C) candida, convexa, rugosa et opalescens. In agaro farinae Zea mays post dies 14 pseudomycelium formantur. Glucosum, galactosum et cellobiosum fermentantur, sucrosus variable at non maltosus at D-xylosus. Glucosum, galactosum, L-sorbosum, maltosum, sucrosus, cellobiosum, trehalosum, D-xylosus, D-arabinosum,

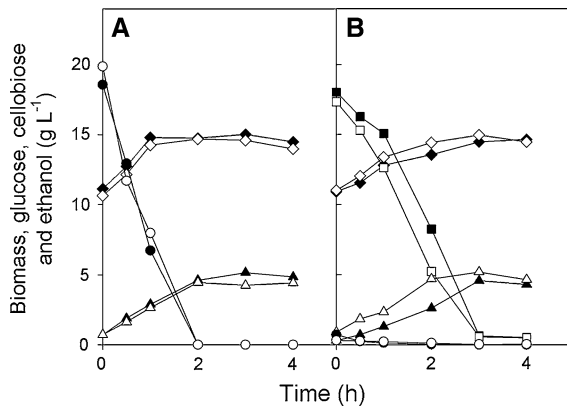


Fig. 3 Sugar batch fermentations by 10 g l^{-1} (dry weight) of *Candida queiroziae* UFMG-CLM-5.1^T (open symbols) or strain UFMG-IMX-6.1 (black symbols) yeast cells in rich YP medium. Biomass (diamonds) and ethanol (triangles) production was determined during the consumption of 20 g l^{-1} of **a** glucose (circles) or **b** cellobiose (squares). In **b** are also shown glucose (circles) concentrations in the medium during cellobiose utilization

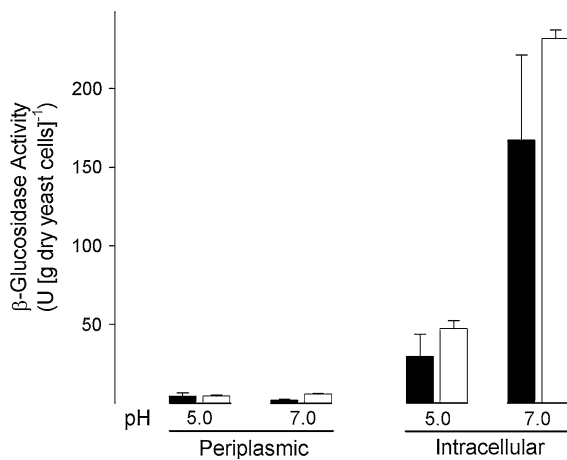


Fig. 4 Cellobiose hydrolysis by a periplasmic or intracellular enzyme was determined in cells of *Candida queiroziae* UFMG-CLM-5.1^T (open bars) or strain UFMG-IMX-6.1 (black bars) collected during growth on 20 g l^{-1} of cellobiose, and the enzymatic activity assayed at the indicated pH

ethanolum, glycerolum, erythritolum, ribitolum (variable), *D*-mannitolum, glucitolum, salicinum, acidum succinicum, acidum citricum, *D*-ribosum (exiguum), hexadecanum (exiguum), xylitolum, acidum lacticum (exiguum), *D*-gluconate et *N*-acetylglucosaminum assimilantur, at non lactosum, melibiosum, raffinolum, melezitolum, inulinum, amyllum solubile, *L*-arabinosum, *L*-rhamnosum, galactitolum, meso-inositolum,

methanolum, *D*-glucosaminum, acetonum, ethyl acetatum nec isopropanolum. Ethylaminum, lysinum et cadaverinum assimilantur at non kalium nitricum et natrium nitrosolum. Augmentum (exiguum) in 37°C . Habitat materiam in Brazil. Typus UFMG-CLM 5.1. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 11853 typus stirps deposita est.

Description of *Candida queiroziae* Santos, Cadete, Badotti, Mouro, Wallheim, Gomes, Stambuk, Lachance & Rosa sp. nov.

In yeast extract (0.5%), glucose (2%) broth after 3 days at 25°C , the cells are ovoid to ellipsoidal ($2\text{--}3 \times 2\text{--}4 \mu\text{m}$). Budding is multilateral (Fig. 5). A sediment is formed after a month, but a pellicle is not observed. On YM agar after 2 days at 17°C , colonies are white, convex, and opalescent. In Dalmau plates after 2 weeks on cornmeal agar, pseudomycelia are formed. Ascospores are not formed. Fermentation of glucose, galactose and cellobiose is positive. Sucrose fermentation is variable. Maltose and *D*-xylose are not fermented. Assimilation of carbon compounds: glucose, galactose, *L*-sorbose, maltose, sucrose, cellobiose, trehalose, *D*-xylose, *D*-arabinose, ethanol, glycerol, erythritol, ribitol (variable), *D*-mannitol, glucitol, salicin, succinic acid, citric acid, *D*-ribose (very weak), hexadecane (very weak), xylitol, lactic acid (very weak), *D*-gluconate and *N*-acetylglucosamine. No growth occurs on lactose, melibiose, raffinose, melezitose, inulin, soluble starch, *L*-arabino-

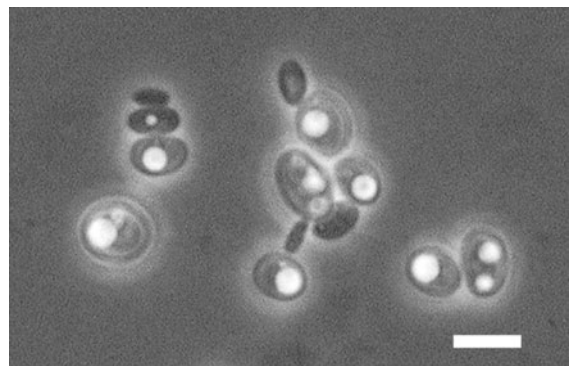


Fig. 5 Phase-contrast micrograph of budding cells of *Candida queiroziae* UFMG-CLM-5.1^T on yeast carbon base agar (Difco) with 0.01% ammonium sulphate after 3 day at 20°C . Bar $5 \mu\text{m}$

L-rhamnose, galactitol, meso-inositol, methanol, D-glucosamine, acetone, ethyl acetate, isopropanol. Assimilation of nitrogen compounds: positive for lysine, ethylamine–HCl, cadaverine, and negative for nitrate and nitrite. Growth in amino-acid-free medium is positive. Growth at 37°C is weak. Growth on YM agar with 10% sodium chloride is positive. Growth in 50% glucose/yeast extract (0.5%) is negative. Starch-like compounds are not produced. In 100 µg cycloheximide ml⁻¹ the growth is positive. Urease activity is negative. Diazonium Blue B reaction is negative. The habitat is rotting wood in Atlantic Rain Forest ecosystem, in the states of Minas Gerais and Rio de Janeiro, Brazil. The type strain accession number of *C. queiroziae* is UFMG-CLM 5.1. It was isolated from rotting wood in Brazil. It has been deposited in the collection of the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as strain CBS 11853^T (=NRRL Y-48722^T). The Myco-bank number is MB 519120. The epithet *queiroziae* (que'í-ro'zi'ae) L. gen. n. *queiroziae*, of Queiroz, in honor to Dr Luzinete Aciole de Queiroz for her contribution to the mycology in Brazil.

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Conflict of interest None

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